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Downregulation and/or Release of NKG2D Ligands as Immune Evasion Strategy of Human Neuroblastoma[1]

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Abstract

Neuroblastoma (NB) is a pediatric extracranial tumor characterized by downregulation of human leukocyte antigen class I and defects of the antigen processing machinery, two features that make it an appropriate target for natural killer (NK)-mediated lysis. NKG2D is an activating immunoreceptor expressed by cytotoxic T lymphocytes and NK cells. The ligands for NKG2D are the major histocompatibility complex class I–related chain (MIC)A and MICB glycoproteins, and the UL-16–binding proteins (ULBPs). Here, the expression of NKG2D ligands was investigated in human primary NB tumors and cell lines because scanty information is available on this issue. MICA, MICB, and ULBP transcripts were found in most tumors and cell lines. MICA protein was detected in some NB cell lines but not in primary tumors. A soluble form of MICA (sMICA) was identified in most patient sera and in some cell line supernatants. sMICA downregulated surface NKG2D in normal peripheral blood $CD8^+$ cells and decreased NK-mediated killing of $MICA^+$ NB cells. MICB was detected exclusively in the cytosol of primary tumors and cell lines. Approximately 50% of primary tumors expressed ULBP-2, but not ULBP-1 or -3. ULBP-3 was expressed in 5 of 9 cell lines, ULBP-2 in 2 of 9, whereas ULBP-1 was never detected. These studies delineate novel potential pathways of tumor escape and immunodeficiency in NB.

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Introduction

Natural killer (NK) cells mediate early innate immune responses against tumor and virally infected cells [1,2]. NK cells kill efficiently transformed cells that have downregulated expression of human leukocyte antigen (HLA) class I molecules [3]. This phenomenon has been explained by the missing-self recognition hypothesis [3]. The activating receptors involved in NK cell–mediated cytotoxicity are the NKp46, NKp44, and NKp30 natural cytotoxicity receptors (NCRs), which belong to the immunoglobulin superfamily [4], and NKG2D.

NKG2D is a C type lectin-like molecule expressed by all NK cells, γ/δ T cells, and CD8 T cells in humans. NKG2D ligands are not expressed in normal cells, but are upregulated upon viral and bacterial infections, transformation, and oxidative stress [5–7]. In humans, NKG2D ligands are the MHC class I–related chain (MIC)A and MICB molecules and the more recently identified family of cytomegalovirus UL-16 binding proteins (ULBPs) [8, 9]. The efficiency of NKG2D-mediated lysis by NK cells has been shown to correlate with the surface density of its ligands on target cells [10]. Recently, it has been reported that soluble MICA (sMICA) and MICB are released in sera from patients with different malignancies and downregulate effector cell–associated NKG2D [11,12].

Neuroblastoma (NB) is the most common extracranial tumor of childhood, often refractory to conventional treatments including surgery, chemotherapy, and radiotherapy [13]. It has been known for a long time that HLA class I antigens are virtually undetectable in NB cell lines and can be upregulated by interferon-γ (IFN-γ) [14,15]. Recently, we have demonstrated multiple defects in the antigen processing machinery of human NB cell lines and primary tumors, suggesting that protocols of T cell–based immunotherapy may be not appropriate for this tumor (L. R., manuscript submitted; Ref. [14]). However, NB cells can represent excellent targets for NK-mediated cytotoxicity, provided that specific activating ligands are expressed on the surface of tumor cells [16].

To the best of our knowledge, scanty information is available about the expression of NKG2D activating ligands in human

Abbreviations: NK, natural killer; HLA, human leukocyte antigen; NCR, natural cytotoxicity receptor; MIC, MHC class I–related chain; ULBP, cytomegalovirus UL-16–binding protein; NB, neuroblastoma; MFI, mean fluorescence intensity; sMICA, soluble MICA; TMB, tetramethylbenzidine; PBMC, peripheral blood mononuclear cells; GD2, disialoganglioside 2

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NB. Only two cell lines were analyzed and both were found to express a MICA$^-$/ULBP$^-$ phenotype [10]. In this study, we have investigated the potential role of NKG2D in immune surveillance against NB by analyzing expression of the various NKG2D ligands on panels of primary tumors and NB cell lines, the release of sMICA, and its impact on recognition of tumor cells by cytotoxic effectors.

Materials and Methods

Patients

This investigation was performed after approval by a local institutional review board.

The criteria used for diagnosis and evaluation of disease extension have been reported elsewhere [17]. Briefly, diagnosis was based on histologic grounds or on bone marrow infiltration by tumor cells, usually associated with elevated urinary catecholamine excretion.

The disease was staged according to the criteria of the International Neuroblastoma Staging System (INSS) [17]. They include the measurement of primary tumor size with ultrasonography and/or computed tomography, a bone marrow study by at least one aspirate, a skeletal study by plain X-ray survey and/or technetium 99^m DPM scintigraphy, and the measurement of urinary vanillylmandelic and homovanillic acids and of serum lactate dehydrogenase [17].

Tissues and Cell Lines

The primary neuroblastic tumors and the sera used in this study were obtained from patients at diagnosis before the implementation of any therapy. An intestinal biopsy was obtained from a patient affected with ulcerative colitis and used for preliminary titration of anti-MICA and anti-MICB monoclonal antibodies (mAbs). Anti-ULBP mAbs were titrated in preliminary experiments performed with human tonsil tissue sections [8].

Tissue samples were fixed in 20% buffered formalin, routinely processed, and embedded in paraffin. One part of some of the lesions was snap frozen in liquid nitrogen and stored at −80°C. For mRNA analysis, primary NB cells were isolated as described [18]. Briefly, NB cell suspensions were first incubated with an anti-GD_2 mAb and then positively selected by immunomagnetic beads coated with anti–mouse immunoglobulin antibodies (Immunotech, Marseille, France), according to the instructions of the manufacturer. The source of the anti-GD_2 mAb (IgG2a) was the supernatant of the ME361-S2a murine hybridoma, purchased from ATCC, Manassas, VA.

The ACN, GI-ME-N, HTLA-230, GI-CA-N, LAN-5, LAN-1, SK-N-BE-2c, SK-N-SH, and SH-SY-5Y neuroblastoma cell lines, the human cervical carcinoma HeLa cell line, the human leukemia U937 cell line, and the human fibroblast 293T strain cell lines were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with L-glutamine, penicillin/streptomycin, nonessential amino acids, and 10% FBS (Sigma) (complete medium). To investigate the release of sMICA, ACN, GI-ME-N, GI-CA-N, and HeLa cell lines were cultured at subconfluence in RPMI 1640 medium without serum for 48 hours. Then the supernatants were collected, concentrated 10 times with Centricon^R Plus-20 (Millipore Corporation, Bedford, MA), and used as indicated.

Monoclonal Antibodies and Flow Cytometry

To characterize the surface and intracellular expression of NKG2D ligands in tumor cell lines, the following mAbs were used: BAM195 (anti-MICA, IgG1; IST, Genova, Italy) [5]; M295 (anti–ULBP-1, IgG1), M311 (anti–ULBP-2, IgG1), and M551 (anti–ULBP-3, IgG1), kindly donated by Dr. David Cosman (Amgen, Seattle, WA) [8]; the mAb clones AUMO1 (anti–ULBP-1, IgG1), BUMO2 (anti–ULBP-2, IgG1), and CUMO2 (anti–ULBP-3, IgM, produced by one of us [A. S.] [11]). The anti-MICB mAb S-JJ5 was generated from a BALB/c mouse immunized with multiple injections of recombinant MICB protein and a peptide corresponding to residues 187 to 203 of MICB. The specificity of mAb S-JJ5 for MICB was shown by its specific reactivity with COS cells transfected with MICB cDNA.

IgG1 or IgM isotype-matched irrelevant mAbs (Southern Biotechnology Associates, Birmingham, AL) were tested as negative controls. All of the experiments were performed using both sets of anti-ULBP mAbs; the results here reported were obtained with the Amgen mAbs because these performed better in immunohistochemical studies.

Phycoerythrin-conjugated AffiniPure F(ab')$_2$ fragments of goat anti–mouse IgG or IgM antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Intracellular staining of cell lines was performed as described [19]. Briefly, cells were washed three times with PBS (Sigma) containing 1% FBS (staining buffer) and fixed with 2% paraformaldehyde at room temperature for 20 minutes. Then, cells were washed twice with staining buffer and incubated in permeabilization buffer (PBS, 1% FBS, 0.1% saponin, Sigma) for 30 minutes at room temperature. Cells (5×10^5 per tube) were next incubated with the primary mAb for 30 minutes at room temperature, then washed twice with permeabilization buffer and incubated with PE-conjugated F(ab')$_2$ fragments of goat anti–mouse IgG or IgM antibodies (Jackson) for 30 minutes at room temperature. Cells were then washed twice in permeabilization buffer and resuspended in staining buffer before being analyzed by flow cytometry using a FACScan instrument (BD Biosciences, San Jose, CA).

For cell surface staining, cells were sequentially incubated with optimal amounts of test mAb and with PE-conjugated F(ab')$_2$ fragments of goat anti–mouse IgG or IgM antibodies, and analyzed by flow cytometry. Isotype- and subclass-matched mouse Ig were used as negative controls in all the experiments. CellQuest software (BD Biosciences) was used for data analysis. The results of flow cytometry experiments are expressed as either mean fluorescence intensity (MFI) or mean relative fluorescence intensity (MRFI), i.e., the ratio between the MFI of cells stained with the selected mAb and the MFI of cells stained with isotype-matched mouse Ig. MFI values of the isotype control and of test mAbs were used

to evaluate whether the differences between the peaks of cells were statistically significant with respect to control. The Kolmogorov-Smirnov test for the analysis of histograms was used, according to the CellQuest software user's guide.

MICA and MICB expression in NB cell lines was also investigated by immunocytochemistry. Cells (2×10^5 per slide) were cytocentrifuged, dried in air, and fixed in 10% buffered formalin for 10 minutes. Endogenous peroxidase activity was blocked by 10-minute incubation at room temperature with methanol containing 3% H_2O_2. Cytospins were then incubated overnight at 4°C with optimal amounts of anti-MICA or -MICB mAbs, or with isotype- and subclass-matched mouse Ig. Cytospins were subsequently washed twice in Optimax Wash Buffer (Menarini Diagnostics, Firenze, Italy) and incubated for 30 minutes at room temperature with Dako Envision System HRP Mouse (Dako, Glostrup, Denmark). After washing in Optimax Wash Buffer, peroxidase activity was detected by incubating cytospins for 6 to 10 minutes at room temperature with Dako Liquid DAB Substrate Chromogen System (Dako). Cytospins were finally counterstained with Mayer's hematoxylin (Sigma).

In some experiments, NB cells were admixed with IL-2–activated NK cells (see below) at a 1:10 ratio, centrifuged, and incubated for 10 minutes at 37°C before being stained for surface and cytosolic MICB and analyzed by flow cytometry.

Reverse Transcription-Polymerase Chain Reaction

RNA was extracted from freshly isolated or cultured cells using RNeasy Mini Kit from Qiagen GmbH (Hilden, Germany) and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) as reported [20]. Primer sequences and profiles of amplification were the following: glyceraldehyde phosphate dehydrogenase (G3PDH) 5′ ACATCGCTCAGAACACCTATGG and 3′ GGGTCTACATGGCAACTGTGAG; CD45 5′ CCTACAGACCCAGTTTCC and 3′ GGCAATCTTTTTCTGTCT; DRβ 5′ CTCCAGCATGGTGTGTCTGA and 3′ GGAGGTTGTGGTGCTGCAGG; MICA 5′ CAGGGACTTGACAGGGAAC and 3′ CCTCTCCTCGGCAAATCCT; MICB 5′ ACCGAGGACTTGACAGAGA and 3′ CCGCTGATGTTTTCTTCT; ULBP-1 5′ TCTGTGCCTCCCGCTTCTG and 3′ GCCTTGGGTTGGGTTGTGC; ULBP-2 5′ GCAACAAGACAGTCACACC and 3′ AGCAGGGGAGGATGATGAG; ULBP-3 5′ TCCATCGGCTTCACACTCA and 3′ GTGAGGTCCAGAGCCAGGT. Amplification profile was 94°C for 1 minute, annealing 60°C (G3PDH), 49°C (CD45), 55°C (DRβ, MICA, and ULBP-3), 59°C (MICB), 57°C (ULBP-1), 53°C (ULBP-2) for 1 minute and extension at 72°C for 1 minute. Each cycle of amplification was repeated 35 times. Ten microliters of each sample was electrophoresed through a 1% agarose gel containing ethidium bromide. The specificity of amplification products was checked by confirming the known base-pair sequence length.

Immunohistochemical Staining of Primary NB Tissues

Immunohistochemical staining of tissue sections was performed using the Envision System horseradish peroxidase (HRP) Mouse (Dako). Briefly, 5-μm-thick sections were cut from formalin-fixed, paraffin-embedded blocks, deparaffinized with xylene, and rehydrated by passages through decreasing concentrations of ethanol (from 100% to 80%). Endogenous peroxidase activity was blocked by a 30-minute incubation at room temperature with methanol containing 3% H_2O_2. Tissue sections were then incubated at 98°C for 40 minutes in citrate buffer (pH 6.0) for antigen retrieval (ChemMate, Dako). After rinsing in Optimax Wash Buffer (Menarini Diagnostics), tissue sections were incubated overnight at 4°C with optimal amounts of test mAbs (anti-MICB, anti-ULBP-1, -2, and -3) or isotype- and subclass-matched mouse Ig. Tissue sections were washed twice in Optimax Wash Buffer and incubated for 30 minutes at room temperature with Dako Envision System HRP Mouse. After washing in Optimax Wash Buffer, peroxidase activity was detected by incubating tissue sections for 6 to 10 minutes at room temperature with Dako Liquid DAB Substrate Chromogen System (Dako). Tissue sections were counterstained with Mayer's hematoxylin (Sigma).

Frozen tissue sections were used as a substrate for immunohistochemical staining with anti-MICA mAb because this mAb did not work on paraffin-embedded, formalin-fixed tissue sections.

Serial tissue sections were stained with NB84 (Dako) and CD45 mAbs (UCHL1, Dako), which detect neuroblasts and cells of hematopoietic origin, respectively. Areas containing at least 80% to 90% neuroblasts were selected for the analysis of MICA, MICB, and ULBP expression. To this end, these areas were first inspected at low magnification and then carefully analyzed at higher magnification (63×).

The percentage of stained tumor cells in each lesion was evaluated independently by two investigators. The variation between the results obtained by these investigators was less than 10%. Results were scored as negative or positive, when the percentage of stained tumor cells in each microscopic area was less than 25% or more than 25%, respectively. The inclusion of each tumor sample in one of the above scores was based on the score of the microscopic area containing the highest percentage of mAb positive neuroblasts.

ELISA

A commercial ELISA Kit by IMMATICS Biotechnologies (Tubingen, Germany) was used to detect sMICA. The assay was performed following the manufacturer's protocol. Briefly, two anti-MICA mAbs binding to different MICA domains were used. Plates were coated with the capture anti-MICA mAb AMO-1 at 2 μg/ml in PBS + 0.1% BSA (Sigma) overnight at 4°C, and blocked by addition of 100 μl of 15% BSA for 2 hours at 37°C. After washings, the standard (recombinant MICA*04 in 0.1% BSA-PBS) and the samples were added and the plates were incubated for 2 hours at 37°C. For analysis of patient sera and cell supernatants, the samples were diluted 1:3 in 0.1% BSA-PBS. After incubation, plates were washed, the detection mAb BAMO-3 at 5 μg/ml in 0.1% BSA-PBS was added, and incubation was continued for 2 hours at 37°C. After washing, an anti–mouse IgG2a-HRP (Santa

Cruz Biotechnology, Santa Cruz, CA), diluted 1:10000 in 0.1% BSA-PBS, was added and incubation was continued for an additional hour at 37°C. Plates were then washed and developed using 3,3′, 5,5′-tetramethylbenzidine (TMB) liquid (Sigma). Absorbance was measured at 450 nm and results were expressed as picograms per milliliter.

Immunoblot Analysis

ACN, GI-ME-N, and HeLa cells were lysed under reducing conditions as described [21]. Briefly, 100-μg protein aliquots were diluted in 250 mM sodium phosphate buffer, pH 8, 2% 50 mM EDTA, and 0.2% sodium dodecyl sulfate (SDS), boiled 5 minutes, subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE), and run in parallel with prestained SDS-PAGE standard (Biorad, Hercules, CA). The resolved proteins were blotted onto nitrocellulose membranes Hybond-C extra (Amersham International, Little Chalfont, Buckinghamshire, UK), and anti-MICB (S-JJ5) mAb was used to localize the corresponding polypeptides on the blots. Peroxidase-conjugated goat anti–mouse antibody was used as secondary antibody (Santa Cruz). Immune complexes were visualized with the use of an enhanced chemiluminescence system (Amersham) according to manufacturer's instructions.

Preparation and Culture of NK-Enriched Cell Suspensions

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient centrifugation of heparinized normal blood samples. PBMCs were washed and resuspended in RPMI 1640 complete medium and depleted of monocytes by adherence to plastic for 1 hour at 37°C. To enrich for NK cells, PBMCs were depleted of T lymphocytes by rosetting with neuraminidase-treated sheep erythrocytes and subsequently incubated with CD14 (Dako) and anti–HLA-DR (D1.12 ATCC) mAbs (30 minutes at 4°C), followed by goat anti-mouse IgG–coated magnetic beads (Immunotech) according to the manufacturer's instructions. The resulting cell fractions, which contained consistently at least 90% $CD3^-$ and $CD56^+$ NK cells, as assessed by double staining with CD3 and CD56 (BD Biosciences) mAbs, were cultured for 4 days at 37°C in complete medium supplemented with 500 U/ml rIL-2 (Proleukin-Chiron Italia s.r.l., Milan, Italy).

Modulation of NKG2D

Normal PBMCs were incubated with $MICA^+$ sera from NB patients or $MICA^-$ sera from healthy donors diluted 1:2 for 24 hours at 37°C. Cells were then stained with anti-NKG2D purified mAb (R&D Systems, Minneapolis, MN) followed by a PE-conjugated goat anti–mouse IgG1 as secondary mAb (BD Biosciences) and with CD8-FITC (BD Biosciences). Cells were finally analyzed by flow cytometry gating on $CD8^+$ cells.

Cytotoxicity Assays

NK cells cultured for 4 days at 37°C with rIL-2 were tested for cytotoxic activity in a 4-hour ^{51}Cr-release assay as previously described [22]. Briefly, $MICA^+$ ACN neuroblastoma cells were labeled with $Na_2^{51}CrO_4$ (Amersham) for 1 hour at 37°C and washed three times. Labeled cells were then added to effector cells in V-bottom microwells at a 40:1 effector to target ratio in a final volume of 200 μl. To assess the interference of sMICA in NKG2D-dependent lysis, activated NK cells were first incubated overnight in the presence or in the absence of sera (1:2 final dilution) from $MICA^+$ NB patients or control sera (1:2 final dilution), or in the presence of NB cell lines supernatants (10× concentrated). In some experiments, a 1-ml aliquot of $sMICA^+$ NB serum or of ACN cell line supernatant was preincubated with immunomagnetic beads (Immunotech), which had been coated with anti-MICA mAb, for 3 hours at room temperature in continuous rotation before being tested in the above assay.

In some experiments, target ACN cells were also incubated for 15 minutes at 37°C with anti-MICA mAb (20 μg/ml) before the addition of effector cells. Chromium release was measured in the supernatants (100 μl) harvested after 4 hours incubation at 37°C. Percent specific lysis was calculated as described [22].

Statistics

Data were analyzed with the Mann-Whitney nonparametric test. A value was considered significant when P was lower than .05.

Results

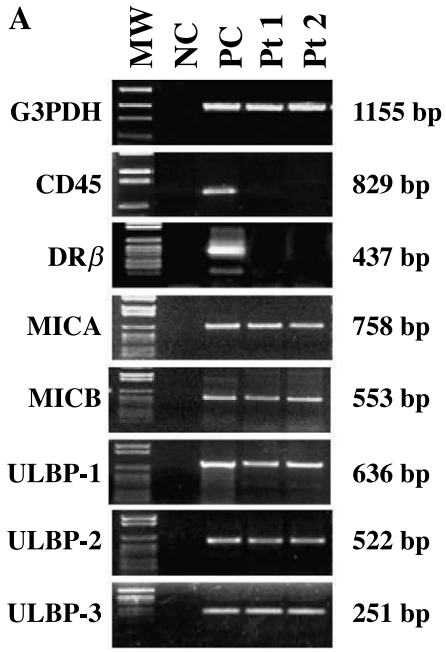
MICA, MICB, ULBP-1, -2, and -3 mRNA Expression in Primary Neuroblasts and NB Cell Lines

RT-PCR analysis of MICA, MICB, ULBP-1, -2, and -3 gene expression was performed on primary neuroblasts and NB cell lines. Primary neuroblasts were isolated from eight tumors (three localized and five disseminated) by positive selection with immunomagnetic beads according to the expression of surface GD2, a disialoganglioside expressed with high specificity on human NB cells [23]. The purity of $GD2^+$ neuroblasts was checked by RT-PCR with CD45 and HLA-DRβ gene-specific primers [18]. Because these genes are not expressed in NB cells, all the following experiments were performed only with $GD2^+$, $CD45^-$ and HLA-$DR\beta^-$ cells (Figure 1, *panel A*). All NB samples consistently expressed MICA, MICB, and ULBP-1 mRNA; ULBP-2 and -3 transcripts were detected in 50% of the cases (Figure 1, *panel B*). With one exception, expression of ULBP-2 and -3 transcripts was observed in the same samples (Figure 1, *panel B*). Figure 1, *panel A*, shows the pattern of MICA, MICB, ULBP-1, -2, and -3 mRNA expression in $GD2^+$ cell fractions from a localized (Pt1) and a metastatic (Pt2) NB tumors.

ULBP-1, -2, -3 and MICB mRNA were detected in 9 of 9, 8 of 9, 8 of 9, and 7 of 9 NB cell lines, respectively (Table 1). In contrast, MICA transcript was expressed only in the SK-N-SH, GI-ME-N, GI-CA-N and ACN cell lines (Table 1).

MICA, MICB, ULBP-1, -2, and -3 Protein Expression in Primary NB Tumors and Cell Lines

Paraffin-embedded tissue sections from 22 newly diagnosed, Schwannian stroma–poor, primary NB were stained



B

NB patients	MICA mRNA	MICB mRNA	ULBP-1 mRNA	ULBP-2 mRNA	ULBP-3 mRNA
1	+	+	+	-	+
2	+	+	-	-	-
3	+	+	+	+	+
4	+	+	+	-	-
5	+	+	+	+	+
6	+	+	+	-	-
7	+	+	+	+	+
8	+	+	+	-	-

Figure 1. *MICA, MICB, ULBP-1, -2, and -3 gene expression in primary GD2$^+$ NB cells as assessed by RT-PCR. In panel A, the results obtained with two representative tumors out of the eight analyzed are shown. Neuroblasts were isolated as GD2$^+$ cells by immunomagnetic bead manipulation. From left to right: MW = molecular weight markers; NC = negative control, represented by water in place of cDNA; PC = positive control, represented by 1) the GD2$^-$ cell fraction isolated from primary NB tumors for CD45 and DRβ gene expression, 2) HeLa cells for MICA gene expression, 3) U937 cells for MICB gene expression, and 4) 293T cells for ULBP gene expression; Pt 1 = NB cells from patient 1; Pt 2 = NB cells from patient 2. The first upper panel shows the amplification product of the G3PDH housekeeping gene tested as control. On the right side of each panel, the expected MWs of the amplified bands are shown. Panel B summarizes the results obtained from the study of the eight primary NB tumors.*

in the immunoperoxidase reaction with an MICB-specific mAb (S-JJ5). Expression of the ULBP proteins was investigated in 12 formalin-fixed, paraffin-embedded tumors. MICA expression was analyzed in frozen tissue sections from eight tumors because BAM195 anti-MICA mAb does not react with formalin-fixed, paraffin-embedded tissues.

Figure 2, *panel A*, shows a representative experiment performed with the anti-MICB mAb, in which most of the tumor cells in every lesion displayed strong cytosolic staining. For comparison, the staining of a representative NB cell line (ACN) with anti-MICB mAb, as assessed by immunocytochemistry, is shown in the inset in panel A. Again, a strong cytosolic staining was obtained using a technique similar to immunohistochemistry.

Figure 2, *panel B*, shows the proportion of NB tumors testing positive for MICB expression. As apparent, such expression did not correlate with the pattern of disease presentation, i.e., localized or metastatic disease.

All NB lesions tested negative for MICA. In these experiments, positive control, represented by an intestinal biopsy from an ulcerative colitis patient, displayed evident MICA staining (data not shown).

ULBP-2 was found to be expressed in approximately 50% of tumors tested, whereas ULBP-1 and -3 were never detected in any lesion. In ULBP-2$^+$ tumors, most cells were stained and the pattern of staining ranged from moderate to intense in the different samples. Figure 2, *panel C*, shows a representative staining of NB tissue with anti–ULBP-2 mAb. The expression of ULBP-2 was unrelated to disease presentation (data not shown). In Figure 2, *panel D*, a negative control stained with an isotype- and subclass-matched irrelevant antibody is shown.

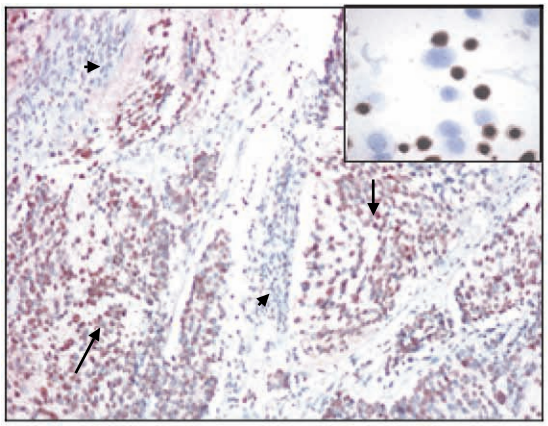
Expression of MICA, MICB, ULBP-1, -2, and -3 proteins was next investigated in a panel of NB cell lines by flow cytometry. MICB was detected on the surface of the U937 cell line tested as positive control, but not on that of any NB cell line (Figure 3, *panels A* and *B*). Conversely, MICB was found in the cytosol of five of these cell lines (Figure 3, *panel A*).

The possibility that MICB could be shuttled from the cytosol to cell surface upon interaction of NB cell lines with IL-2–activated NK cells was investigated using the ACN cell line. However, these experiments showed that no surface MICB expression was detectable under these conditions (data not shown).

Table 1. MICA, MICB, ULBP-1, -2, and -3 mRNA Expression by NB Cell Lines.

NB cell lines	MICA	MICB	ULBP-1	ULBP-2	ULBP-3
HTLA-230	–	+	+	+	+
SK-N-SH	+	+	+	+	+
SH-SY-5Y	–	–	+	+	+
SK-N-BE-2c	–	+	+	–	–
ACN	+	+	+	+	+
GI-CA-N	+	+	+	+	+
GI-ME-N	+	+	+	+	+
LAN-5	–	–	+	+	+
LAN-1	–	+	+	+	+

A

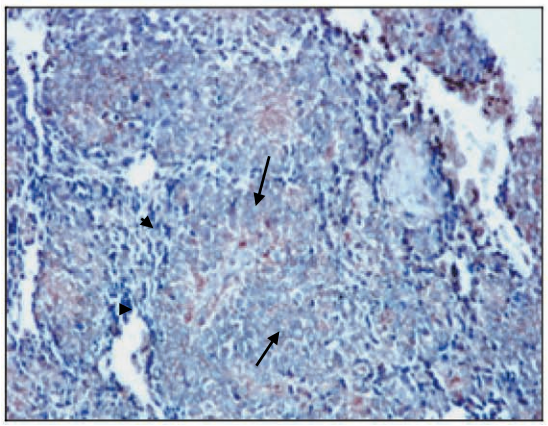


B

NB stages	Score▪	MICB expression	
		N°	%
Disseminated	-	3 (17)	18
	+	14 (17)	82
Localized	-	0 (5)	0
	+	5 (5)	100

▪positve, (> 25%), -, negative (< 25%)

C



D

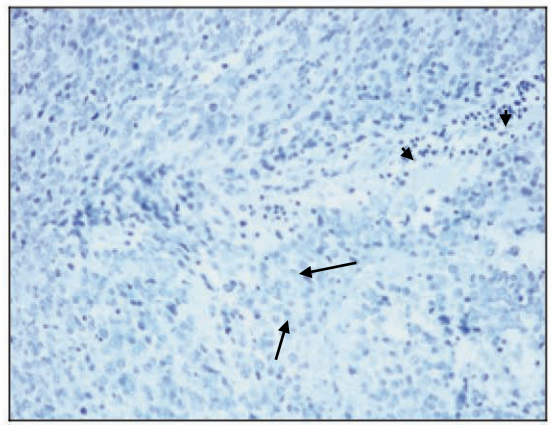


Figure 2. *MICB and ULBP-2 protein expression in primary human NB tumors. Immunoperoxidase staining of formalin-fixed, paraffin-embedded NB primary tumors. Panel A shows one representative staining for anti-MICB mAb (S-JJ5). Inset panel shows a representative staining of ACN NB cell line for anti-MICB mAb by immunocytochemistry. Panel B summarizes the data obtained from immunohistochemical analysis of 17 disseminated and 5 localized NB primary tumors. Panel C shows one representative staining for anti-ULBP-2 mAb (M311). Panel D shows negative control stained with an isotype- and subclass-matched irrelevant antibody. Arrows indicate neuroblasts stained by the above mAbs, arrowheads indicate negatively stained lymphoid infiltrates. Original magnification, ×40.*

To prove unambiguously that staining of NB cells by the MICB-specific mAb S-JJ5 reflected reactivity with MICB protein, lysates of the NB cell lines ACN and GI-ME-N were tested with mAb S-JJ5 in Western blotting. The cell line U937 was used as a positive control. All cell lines showed a band of 43 kDa and two fainter bands of 45 and 55 kDa. These molecular forms of the MICB protein fall in the range reported by Dunn et al. [24]. Figure 3, *panel C*, shows a representative experiment out of the three performed, in which ACN NB cells and U937 cells were tested. These results indicate the MICB bands detected by Western blot were the same in NB cell lines, which retain the protein in the cytosol, and in U937 cells, which express it on the cell surface.

MICA was not detected either in the cytosol or on the surface of any NB cell line, with the exception of ACN and GI-CA-N cells, which, in analogy to HeLa cells tested as positive control (data not shown), expressed surface MICA at high intensity (Figure 3, *panel B*). ACN and GI-CA-N cells also displayed intracellular staining for MICA (data not shown). Similar patterns of MICA expression were observed when cell lines were stained by immunocytochemistry (not shown).

ULBP-2 was detected on the surface of the GI-CA-N and GI-ME-N cells, ULBP-3 on the surface of HTLA-230, SK-N-SH, ACN, GI-CA-N, and GI-ME-N cells (Figure 3, *panel B*). Cell lines testing negative for surface expression of ULBP-2 or -3 did not express these ligands intracellularly. ULBP-1 was never detected either on the surface or in the cytosol of any cell line (Figure 3, *panel B*). All anti-ULBP mAbs stained the cell surface of the 293T cell line tested as positive control (data not shown).

In conclusion, the above data demonstrated downregulation of the ligands for the NKG2D-activating receptor on the surface of primary neuroblasts and NB cell lines, suggesting that tumor cells can often escape from the control of NKG2D$^+$ cytotoxic effectors.

Release of sMICA by NB Tumors

Next, MICA release in NB patient sera was investigated by ELISA. Serum samples of 51 healthy donors and 20 NB patients were tested. Thirteen of the latter sera were from patients with metastatic disease, whereas 7 were from patients with localized disease.

The majority of sera from normal individuals contained levels of sMICA close to the detection limit of the ELISA (10 pg/ml), with a median of 10 pg/ml (Figure 4, *panels A* and *B*). sMICA in sera from NB patients ranged between

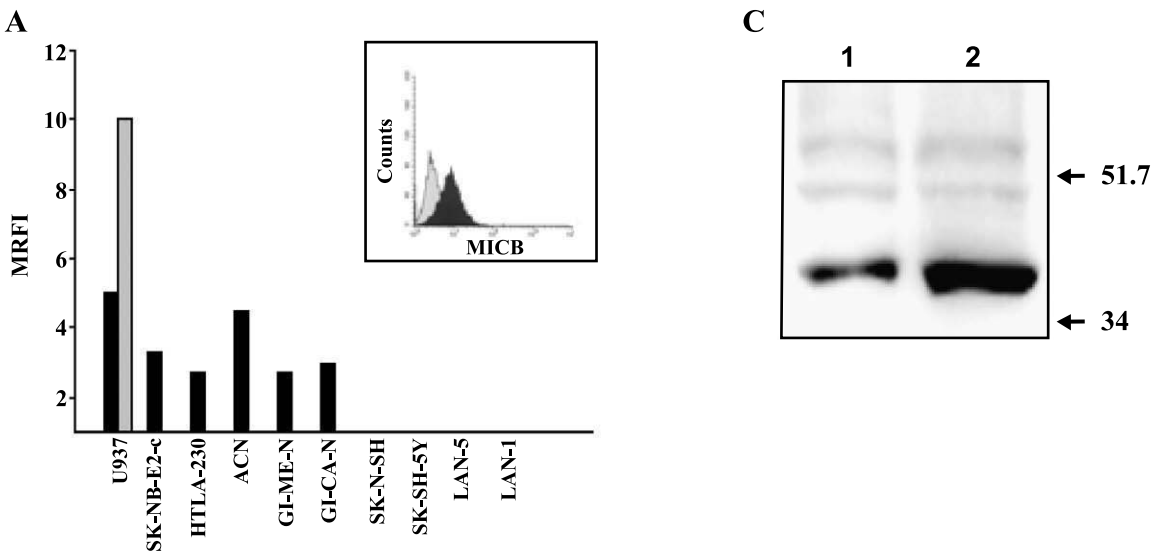
10 and 1470 pg/ml, with a median of 120 pg/ml (Figure 4, *panels A* and *B*). The differences between the concentrations of sMICA in sera from healthy donors and NB patients were statistically significant ($P < .0001$), as determined by Mann-Whitney nonparametric test (Figure 4, *panel A*). No correlation was found in NB patients between sMICA serum concentrations and disease presentation.

The presence of sMICA was also evaluated in concentrated culture supernatants of NB cell lines. sMICA at the concentration of 200 and 85 pg/ml, respectively, was detected only in supernatant of ACN and GI-CA-N cell lines, after 48 hours culture at 37°C in serum-free medium.

Downregulation of NKG2D and Inhibition of NKG2D-Mediated Cytolytic Activity by sMICA

We subsequently investigated the effects of sMICA on NKG2D expression in normal PBMCs. The latter cells were cultured in the presence of $sMICA^+$ serum from an NB patient or $sMICA^-$ serum from a healthy donor. As shown in Figure 5, NKG2D expression on $CD8^+$ cells, which contain most of the $NKG2D^+$ cells in human peripheral blood, was consistently reduced by PBMC incubation with serum of a NB patient containing 740 pg/ml sMICA, but not with normal serum.

To address the functional role of sMICA with regard to NKG2D-mediated cytolytic activity, we performed cytotoxicity



B

NB cell lines	MICA	MICB	ULBP-1	ULBP-2	ULBP-3
HTLA-230	1¤	1	1	1	4.5
SK-N-SH	1	1	1.5	1.8	6
SH-SY-5Y	1	1	1	1	1
SK-N-BE-2c	1	1	2	2	2
ACN	70	1	1	1	5
GI-CA-N	16	1	1	3,8	16,5
GI-ME-N	1	1	1	4,5	13,8
LAN-5	1	1	1	1	1
LAN-1	1	1	1	2	1.5

¤Values indicated are expressed as MRFI, i.e the ratio between the MFI of cells stained with the selected mAb and the MFI of cells stained with the isotype-matched mouse Ig. Those considered significantly high are underlined.

Figure 3. *Surface and intracellular expression of MICB in human NB cell lines. (Panel A) The cell lines were stained with the anti-MICB mAb (S-JJ5) and subsequently analyzed by flow cytometry as detailed in the Materials and Methods section. One representative experiment out of the three performed with similar results is shown. Gray histograms represent surface staining and black histograms represent intracellular staining for MICB. U937 cells are shown as positive control for MICB surface expression. Results are expressed as MRFI. The inset in the right upper side of the figure shows a representative histogram obtained by intracellular staining of SK-N-BE-2c cells. The gray profile indicates staining with isotype-matched control mAb, whereas the black profile refers to staining with anti-MICB mAb. Panel B summarizes the results of surface staining of the whole panel of NB cell lines with mAbs to all NKG2D ligands. (Panel C) Western blot analysis of lysates from ACN NB cells (lane 1) and U937 human leukemia cells (lane 2) tested as positive control with anti-MICB mAb (S-JJ5). The position of molecular weight markers is on the right side. One representative experiment out of the three performed is shown.*

assays using IL-2–activated NK cell populations that had been isolated from different healthy donors as effectors, and the surface MICA$^+$ ACN NB cell line as target. As shown in the representative experiment of Figure 6, ACN cells were efficiently lysed by NK cells (70% specific lysis at a 40:1 effector to target ratio). Cytotoxicity was partly dependent on the MICA-NKG2D interaction because it was consistently reduced (to 45% specific lysis) by target cell incubation with anti-MICA mAb (BAM195) (Figure 6), but not with control mAb (data not shown). Likewise, inhibition of NKG2D-mediated lysis was observed when NK cells were preincubated with serum of NB patient containing 720 pg/ml sMICA (40% specific lysis), but not with sMICA$^-$ normal serum (68% specific lysis) (Figure 6). This inhibitory effect was abolished when sMICA$^+$ serum of NB patient was preincubated with anti-MICA mAb bound to immunomagnetic beads (72% specific lysis) (Figure 6).

In contrast, no inhibition was observed when sMICA$^+$ serum of the same NB patient was preincubated with an isotype- and subclass-matched mAb of irrelevant specificity bound to immunomagnetic beads (data not shown).

sMICA$^+$ supernatant from ACN NB cells inhibited NKG2D-mediated lysis to a lesser extent (53% specific lysis, data not shown) than NB patient serum. In contrast sMICA$^-$ supernatant of HTLA-230 NB cells did not effect NK-mediated lysis (70%, data not shown).

These studies indicate that sMICA present in sera of NB patients can impair NKG2D expression on cytotoxic cells and their effector function against NKG2D ligand expressing target cells.

Discussion

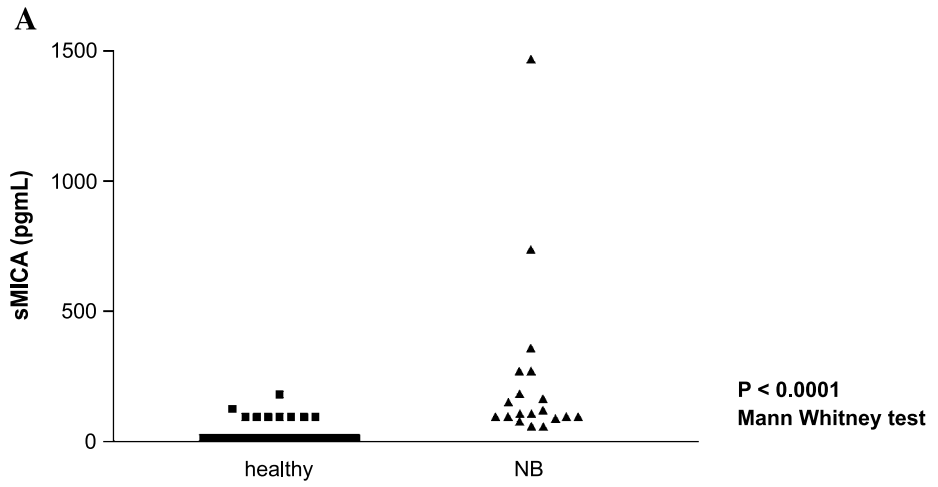
Human NB cells display low to absent expression of surface HLA molecules, thus escaping specific T-cell recognition [14,15,25]. This feature makes them an attractive target for NK cell–based immunotherapy.

The NK sensitivity of NB cell lines has been known for a long time, but recent studies have shed new light on the mechanisms involved [26]. In particular, lysis of NB cells by NK cell lines was found to depend on NCR expression on the latter cells, and pretreatment of effector cells with a cocktail of anti-NCR mAbs strongly reduced cytotoxic activity [16].

In this study we provide the first phenotypic and functional characterization of the major ligands of NKG2D, another activating receptor of cytotoxicity, in human NB.

Most primary NB tumors showed MICA and MICB expression both at the mRNA and protein levels, and similar patterns were observed in a panel of NB cell lines. The MICA protein was not detected in tumor cells, but was present in soluble form in most NB sera; in contrast, the MICB protein was found consistently in the cytosol, but not on the surface of tumor cells. These patterns of MICA/MICB expression differ from those observed in non-tumor-bearing individuals. Thus, in the latter, MICA/MICB surface expression is induced by inflammatory stress in intestinal epithelial cells [5, 7], and soluble MICA and MICB are detected in serum only occasionally and at very low levels [11].

Previous studies have identified sMICA in serum from patients with different malignancies [11, 12]. In view of the mechanism of sMICA production, operated by metalloproteinase-dependent proteolytic cleavage [27], failure to detect



B

	Lowest pg/mL	Highest pg/mL	Median pg/mL	Mean pg/mL	Std error pg/mL	N° of Samples
Healthy donors	10	184	10	27	5	51
NB patients	10	1470	120	240	73	20

Figure 4. *Levels of sMICA (pg/ml) in sera of NB patients and healthy donors (panel A). Serum samples of 20 NB patients and 51 healthy donors were tested by ELISA. Panel B shows the lowest and highest values detected in both groups together with median, mean, and standard error.*

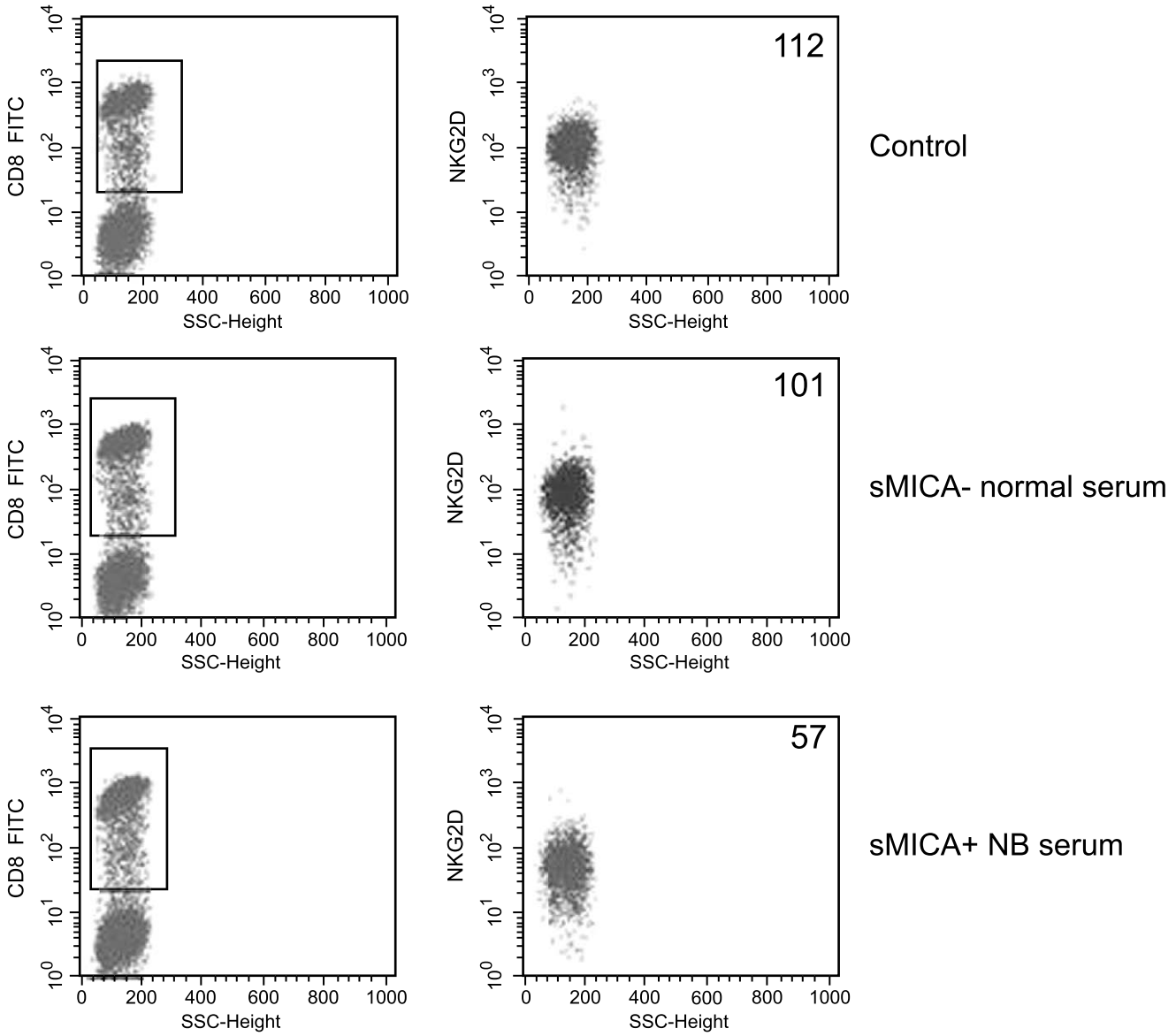


Figure 5. *Modulation of NKG2D expression on peripheral blood CD8⁺ cells from normal donors by sMICA⁺ serum from a NB patient. One representative experiment out of the three performed is shown. The fluorescence intensity of NKG2D was measured on PBMCs from a healthy donor that had been untreated (control: upper dot plot panel), incubated with sMICA⁻ normal serum (middle dot plot panel) or with sMICA⁺ serum of an NB patient (lower dot plot panel). NKG2D expression was analyzed gating on CD8⁺ cells (boxed areas in plots on the left side of the figure). Numbers in the top right corners of plots on the right side indicate NKG2D MFI (subtracted from background). MFI of IgG1 isotype negative control was 9 (data not shown).*

MICA expression in primary tumors may appear somewhat unexpected. However, it is conceivable that newly formed surface or cytosolic MICA is cleaved very quickly to generate the soluble form, thus hindering MICA detection in tissue sections. An additional factor that may contribute to the latter phenomenon is the limited sensitivity of immunohistochemistry. This hypothesis is supported by the finding that both NB cell lines releasing sMICA in culture supernatants showed surface and cytosolic MICA expression, as assessed by the more sensitive flow cytometry.

In this study, functional experiments demonstrated that sMICA in patient sera downregulated NKG2D expression on the surface of $CD8^+$ cells and reduced cytotoxicity of IL-2–activated NK cells against $MICA^+$ target cells. These results are consistent with various reports in different tumors [11, 12] and point to a general strategy of immune evasion used by malignant cells. Furthermore, other NKG2D ligands besides MICA may be released in soluble form. Soluble MICB has been detected in sera from patients with hematological malignancies [11], and ULBP-1, whose transcript was found here to be expressed in most primary tumors, may be another candidate soluble molecule.

A novel potential strategy of immune evasion delineated in the present investigation is the cytosolic retention of MICB observed in primary NB tumors and cell lines. Thus, failure of NB cells to express MICB on the cell surface would limit their interactions with $NKG2D^+$ cytotoxic cells.

A similar phenomenon has been demonstrated in human tumor cell lines transfected with the UL16 cytomegalovirus gene [24]. The UL16 protein caused MICB retention in the

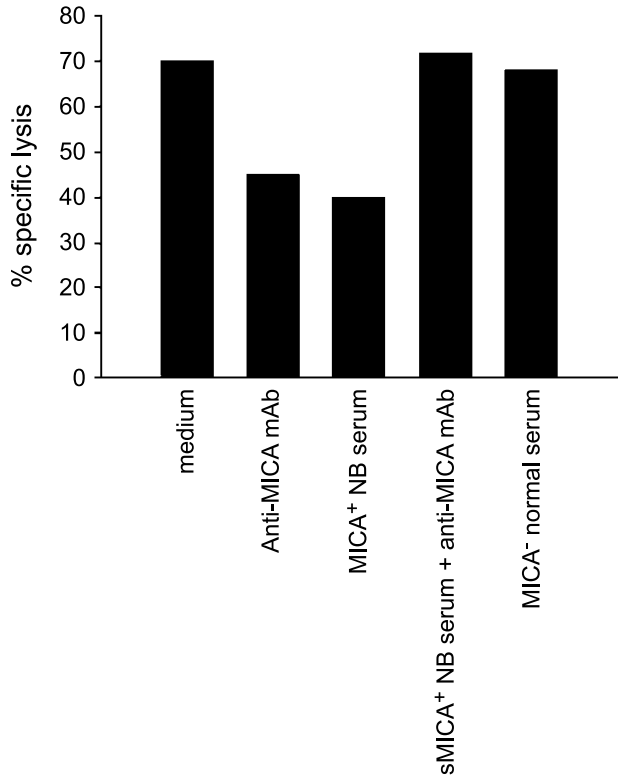


Figure 6. *Reduction of NKG2D-mediated killing of NB cells by soluble MICA. The surface MICA$^+$ ACN NB cell line was tested as target of normal activated NK cells in a 4-hour chromium release assay. NKG2D-dependent cytolytic activity was evaluated by preincubating effector cells with medium, anti-MICA mAb, sMICA$^+$ serum of a NB patient, sMICA$^+$ serum of the same NB patient pretreated with anti-MICA mAb bound to immunomagnetic beads, or sMICA$^-$ normal serum. The figure shows one representative experiment out of the three performed.*

endoplasmic reticulum and the cis-Golgi apparatus, where it inhibited the processing of MICB to mature forms [24].

The mechanism(s) involved in cytosolic sequestration of the MICB protein in NB cells is so far unknown. Our preliminary results indicate that the molecular weight of MICB was similar in NB cell lines and in U937 cells, irrespective of MICB expression in the cytosol of the former and on the surface of the latter cells. These findings suggest that cytosolic MICB retention in NB cells may be consequent to altered intracellular trafficking of the protein, but this issue warrants further investigation.

The only NKG2D ligand expressed on the surface of primary NB tumors is ULBP-2, which was detected in half of the cases, whereas ULBP-1 and ULBP-3 staining was consistently negative. In NB cell lines, ULBP-3 was detected in half of the cell lines, ULBP-2 in a small number of them, and ULBP-1 in none. The discrepancies in the patterns of ULBP expression between primary tumors and cell lines may be related to changes induced by long-term culture in the latter cells or to the different sensitivity of the detection techniques used. Based on the current knowledge, no specific functions of individual ULBP proteins for interaction with NK are known.

In conclusion, NB cells use multiple mechanisms to evade the control of the host immune system. These include 1) downregulation of MICA expression associated with production of sMICA, 2) cytosolic sequestration of MICB, and 3) downregulation of ULBP molecules. It is conceivable that these very same mechanisms contribute to the pathogenesis of immune deficiency in NB patients, since functional impairment of NKG2D may reduce the efficacy of NK cell and CTL responses against target cells infected by pathogens, especially viruses.

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